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Both homo and heterodimers of Marek's disease virus encoded Meq protein contribute to transformation of lymphocytes in chickens

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ABSTRACT

Marek' disease virus serotype-1, also know as *Gallid herpesvirus 2* (GaHV-2), elicits T-cell lymphomas in chickens. The GaHV-2 genome encodes an oncoprotein, Meq, with similarity to the Jun/Fos family of proteins. We have previously shown that Meq homodimers are not sufficient to induce lymphomas in chickens. In this study, we investigated the role of Meq heterodimers in the pathogenicity of GaHV-2 by generating a chimeric *meq* gene, which contains the leucine zipper region of Fos (*meqFos*). A recombinant virus containing the *meqFos* gene in place of parental *meq*, rMd5-MeqFos, was not capable of transforming chicken lymphocytes, indicating that heterodimerization of Meq alone is not sufficient for transformation. In addition, the recovery of the oncogenic phenotype by a recombinant virus encoding one copy each of MeqGCN (homodimer) and MeqFos (heterodimer) conclusively demonstrates that both homo and heterodimerization of Meq are required for oncogenesis.

Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by *Gallid herpesvirus-2* (GaHV-2) which, together with closely related *Gallid herpesvirus-3* (GaHV-3) and *Meleagrid herpesvirus-1* (MeHV-1) is classified in the genus *Mardivirus* within the subfamily *Alphaherpesvirinae* (ICTVdB-Management, 2006).

The highly oncogenic nature of GaHV-2 suggested the presence of a viral oncogene(s). Genome sequence analysis confirmed the presence of a putative oncogene, *meq*, unique to GaHV-2. The *meq* gene is named after the EcoQ fragment where it is located, "*M*arek's *EcoQ*", and two copies are found in the viral genome within the terminal repeat long (TRL) and internal repeat long (IRL) regions (Peng et al., 1992; Ross et al., 1997; Ross, 1999; Silva et al., 2001; Tulman et al., 2000). Meq is a 339 amino acid nuclear phosphoprotein that is abundantly expressed in transformed cells. The structure of Meq is well defined. Meq contains nuclear and nucleolar localization signals, a bZIP (basic-region leucine zipper) domain and a proline rich transactivation/repression domain (Kung et al., 2001; Liu et al., 1997; Qian et al., 1996). The bZIP domain of Meq shares significant homology, with the protooncogene c-Jun, a transcription factor of the AP-1 (activating protein) complex (Jones et al., 1992). AP-1 transcription factors are well-described proteins that include the Jun, Fos and ATF/CREB families. AP-1 proteins are characterized by their ability to bind and regulate sequence specific gene elements, AP-1 sites, which are found in many genes associated with cell proliferation (Shaulian and Karin, 2001). Transformation by deregulated expression of c-Jun, c-Fos and their viral counterparts v-Jun and v-Fos is well documented. The shared homology between Meq and c-Jun suggests Meq may transform through similar mechanisms (Milde-Langosch, 2005; Vogt, 2001; Vogt and Bader, 2005).

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A considerable amount of *in vitro* data supports the oncogenic nature of Meq, which has been shown to promote transformation, anchorage-independent growth, cell-cycle progression, and anti-apoptotic activity (Ajithdoss et al., 2009; Liu et al., 1998, 1999). More recently, *in vitro* expression of Meq was shown to upregulate genes similar to those upregulated by v-Jun, suggesting that Meq transforms *via* a v-Jun transforming pathway (Levy et al., 2005). The conclusive role of Meq in transformation was confirmed by the generation of a Meq-negative recombinant virus, which failed to cause tumors in infected chickens. (Lupiani et al., 2004). The essential role of Meq in GaHV-2 transformation has been further supported by generation of Meq mutant viruses, such as Meq C-terminal binding mutant (pRB-1B-Ct20) and more recently Meq homodimer mutant virus (rMd5-MeqGCN) both of which are non-oncogenic (Brown et al., 2006; Suchodolski et al., 2009).

It has been shown that the leucine zipper region of bZIP proteins determines the dimerization partners of bZIP proteins, which in turn are important determinants of DNA binding specificity and therefore transcriptional regulation. For example, different c-Jun dimers have been shown to play distinct roles in transformation, i.e. anchorage or serum independent growth (Vogt, 2001). Like c-Jun, Meq can both homodimerize and heterodimerize and has the ability to dimerize with c-Jun, Fos, CREB and ATF members. The DNA binding properties of Meq depend on its dimerization partner (Levy et al., 2003). Previous characterization of the *in vitro* DNA binding properties of Meq revealed Meq/c-Jun heterodimers bind AP-1 sequences with greater affinity than Meq homodimers (Levy et al., 2003; Qian et al., 1996). In addition, Meq and c-Jun were shown to bind AP-1 sequences, but only Meq was shown to bind sequences contained in the GaHV-2 origin of replication (Ori). Transcriptional analysis of Meq on the AP-1-like containing *meq* promoter, and the lytic *pp14/38* bidirectional promoter which contains the GaHV-2 Ori, revealed that Meq transactivates the *meq* promoter but represses the bidirectional *pp14/38* promoter (Levy et al., 2003). Meq also has been shown to bind the immediate early ICP4 promoter region by chromatin immunoprecipitation analysis therefore potentially regulating other lytic genes (Levy et al., 2003). This, together with luciferase reporter data, suggests that Meq heterodimers activate AP-1 containing promoters, therefore potentially activating genes associated with cell proliferation, while Meq homodimers may repress genes associated with lytic infection and consequently may be involved in regulating lytic or latent infection. Collectively, these data point to a role of Meq heterodimers in transformation and Meq homodimers in regulation of viral replication.

In order to delineate the functions of Meq and address the role of Meq homodimers in GaHV-2 pathogenesis, we recently reported a recombinant Meq mutant virus (rMd5-MeqGCN) expressing a chimeric *meq* gene (*MeqGCN*), which allowed for only Meq homodimer formation. Although this virus replicated *in vitro* and *in vivo*, the ability to transform lymphocytes was lost, supporting a role for Meq heterodimers in transformation (Suchodolski et al., 2009).

In order to evaluate the role of Meq/c-Jun heterodimers in GaHV-2 pathogenesis, we constructed a recombinant virus that predominantly allowed for the formation of Meq/c-Jun heterodimers by substituting the parental Meq leucine zipper with the leucine zipper of c-Fos (MeqFos). The replication of rMd5-MeqFos during early cytolytic infection in lymphoid organs was not affected, however, infected chickens did not develop gross tumors. These results are in agreement with those recently reported for another Meq mutant virus that does not homodimerize but heterodimerizes with c-Jun resulting in complete loss of oncogenicity, suggesting that heterodimers alone are not sufficient for transformation of lymphocytes (Brown et al., 2009). In addition, our studies with a double mutant virus, rMd5-MeqFos/GCN, which contains one copy of *meqGCN* gene (homodimer) and one copy of *meqFos* (heterodimer) provide the first *in vivo* evidence that both homo and heterodimers are involved in complementing pathways in GaHV-2 pathogenesis as this mutant that was able to transform lymphocytes.

Results

Mutations in the leucine zipper of Meq are sufficient to confer heterodimerization with c-Jun

It is well documented that the specific amino acid residues within the leucine zipper of bZIP proteins determine their dimerization properties. Among these transcription factors, c-Fos has been extensively studied and it is known that its leucine zipper exclusively forms heterodimers with the Jun family of transcription factors (Hai and Curran, 1991; Kouzarides and Ziff, 1989; Ransone et al., 1990). Similar to other studies (Castellazzi et al., 1993; Hai and Curran, 1991; Hartl and Vogt, 1992; Jurdic et al., 1995; Ransone et al., 1990) we utilized the leucine zipper region of c-Fos in place of the parental leucine zipper of Meq to study the function of Meq/Jun heterodimers in GaHV-2 pathogenesis. The leucine zipper of *meq* was successfully "swapped" with the leucine zipper of *c-fos* using overlapping PCR, resulting in a Meq heterodimer mutant, MeqFos (Fig. 1).

Co-immunoprecipitation experiments were performed to test the dimerization of MeqFos by transfecting 293 T cells with pcDNA vectors expressing Flag and HA tagged Meq, MeqFos, and c-Jun constructs. Cell lysates were immunoprecipitated with anti-FLAG antibody followed by Western blot analysis to evaluate if the presence of HA-tagged proteins were in the dimer complex. As demonstrated in

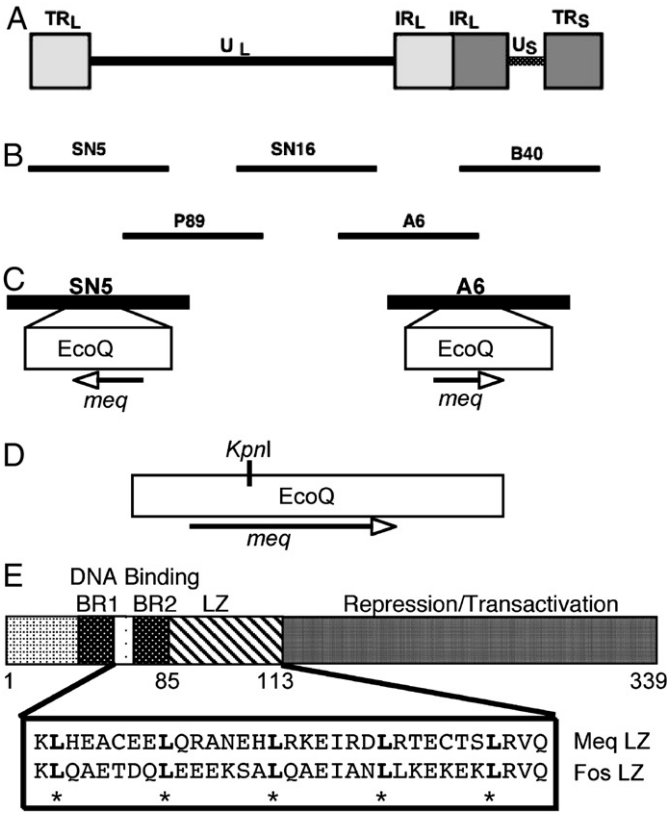

Fig. 1. Cosmid clones used to recover recombinant viruses. (A) Organization of the serotype 1 MDV genome. (B) Schematic representation of the overlapping cosmid clones used to reconstitute recombinant viruses, rMd5 and rMeqFos, derived from a very virulent (vv) strain of MDV (Md5). (C) Location of EcoQ fragment and Meq gene in cosmids SN5 and A6. (D) Location of *Kpn*I site described in Materials and methods, located within the EcoQ fragment at nucleotide 385 of the *meq* gene. (E) Schematic representation of the Meq protein and Meq and Fos leucine zippers (LZ). The DNA binding basic regions (BR1 and BR2) and transactivation domains, are depicted. The LZ sequence of parental Meq and c-Fos used to replace parental Meq LZ in rMd5-MeqFos are shown. Asterisks indicate the conserved leucine sites.

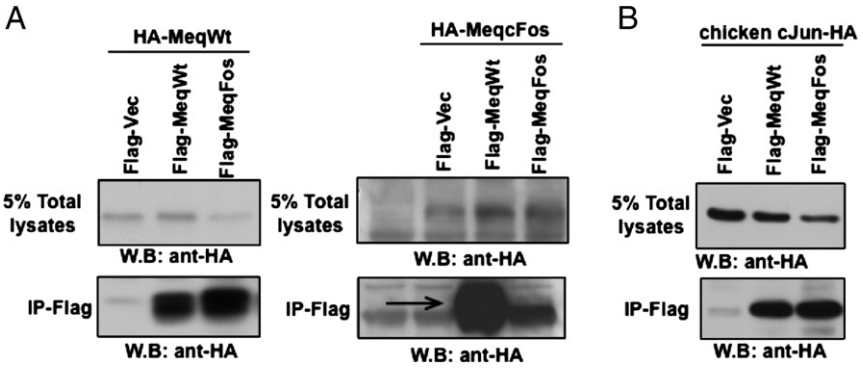

Fig. 2. Heterodimerization of Meq and MeqFos. (A) Co-immunoprecipitation analysis of tagged Meq and MeqFos proteins. 293 T cells were transfected with the indicated expression plasmids. Five percent of the total cell lysates that were used for co-immunoprecipitation were also included as controls. MeqFos heterodimerizes with wild type (wt) Meq (left panel) but does not homodimerize (right panel and arrow). (B) MeqFos retains ability to form heterodimers with c-Jun. The indicated expression plasmids were cotransfected in 293 T cells. Five percent of the total cell lysates that were used for immunoprecipitation were also included in the same gel as a control. Both Meq and MeqFos effectively precipitate with c-Jun.

Fig. 2A, HA-Meq co-precipitated with Flag-Meq and Flag-MeqFos, however, only a small amount of HA-MeqFos co-precipitated with Flag-MeqFos, therefore demonstrating MeqFos preferentially forms heterodimers. Co-immunoprecipitation was also performed to confirm the ability of MeqFos to dimerize with c-Jun. Cells were transfected with Flag-Meq and Jun-HA as a positive control, and Flag-MeqFos and Jun-HA. As shown in Fig. 2B both Flag-Meq and Flag-MeqFos effectively co-precipitate with Jun-HA, therefore verifying dimerization of MeqFos with c-Jun.

Meq heterodimer mutant (MeqFos) retains DNA binding, transactivation and repressive functions

Previous work has shown that Meq homodimers and heterodimers have different DNA binding affinities. Although both Meq homodimers and heterodimers bound AP-1 sequences, Meq together with c-Jun bound AP-1 sequences with greater affinity than Meq alone. In addition, Meq alone bound the GaHV-2 Ori located in the *pp38/14* bidirectional promoter by EMSA analysis using bacterial expressed proteins (Levy et al., 2003). Luciferase reporter assays have also shown functional differences between Meq and Meq plus c-Jun in that although Meq activated the AP-1 containing *meq* promoter, Meq plus c-Jun resulted in higher activation. Furthermore, Meq expression was shown to repress the *pp38/pp14* bidirectional promoter that contains the GaHV-2 Ori (Levy et al., 2003). Therefore, luciferase assays were performed to test the DNA binding and transactivation/repression functions of MeqFos.

Luciferase assays were utilized to test the transactivation or repressive functions of MeqFos compared to parental Meq. DF-1 cells were transfected with pcDNA empty vector, pcDNA-Meq, pcDNA-MeqFos, pcDNA-c-Jun, or a combination of pcDNA-Meq, pcDNA-MeqFos, and pcDNA-c-Jun, together with reporter vectors pGL3-*meq*, pGL3-*pp14*, pGL3-*pp38*, or pGL2-3X-Ori. As shown in Fig. 3A, Meq activated the *meq* promoter, however MeqFos required co-transfection with pcDNA-c-Jun for transactivation, therefore indicating heterodimerization of MeqFos with c-Jun is required for transactivation. Since Meq homodimers were previously shown to bind the GaHV-2 Ori, which is located in the *pp38/14* promoter, it was speculated that Meq homodimers mediated repression of the *pp38/14* promoter (Levy et al., 2003). However, our results show that both Meq and MeqFos repressed *pp38* and *pp14* promoters and the pGL2-3X-Ori reporter, which contains three copies of the GaHV-2 Ori (Fig.

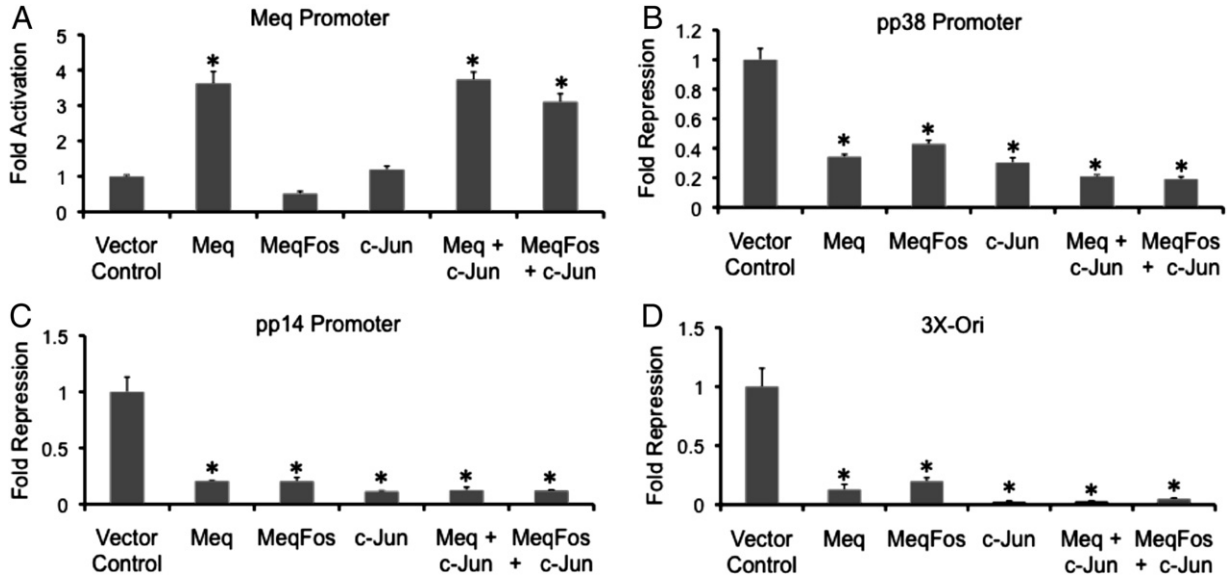

Fig. 3. Luciferase assays demonstrate that MeqFos has functional transactivation and repression activities. (A) DF-1 cells were transfected with *meq*-promoter, (B) *pp38* promoter, (C) *pp14* promoter or (D) 3X-Ori luciferase reporter plasmids and pcDNA (empty vector), pcDNA-Meq, pcDNA-MeqFos, or pcDNA c-Jun, with or without c-Jun. Both Meq and MeqFos activate the *meq promoter* and repress the *pp38/14* bidirectional promoters and 3X-Ori. However, activation of the *meq* promoter by MeqFos requires c-Jun. Luciferase values are expressed as fold difference relative to pcDNA vector. Significance (*) equals $p \leq 0.05$. Error bars indicate SD.

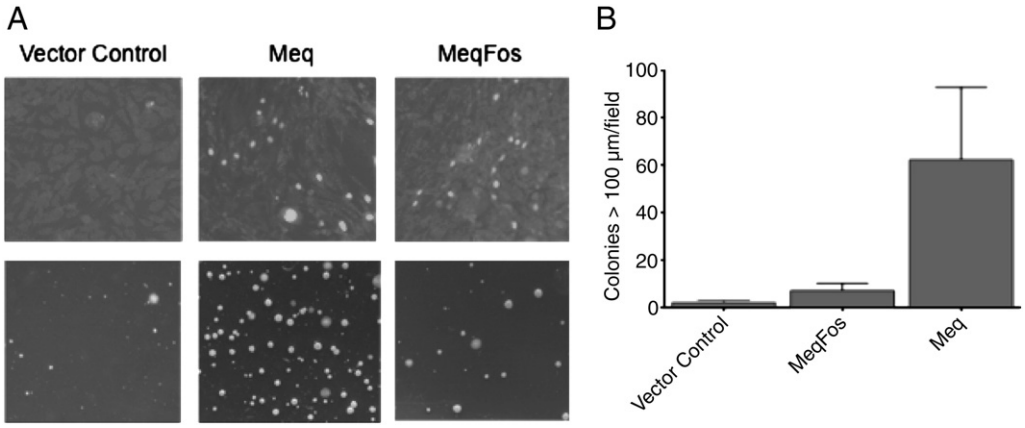

Fig. 4. *In vitro* soft agar assays demonstrating that MeqFos has reduced transformation potential. (A) Top panel: immunofluorescence analysis of Meq expression from pools of selected DF-1 cells transfected with pcDNA, pcDNA-Meq, pcDNA-MeqFos. Bottom panel: Soft agar assay was performed using pcDNA, pcDNA-Meq, pcDNA-MeqFos selected DF-1 cells to assess anchorage independent growth. (B) Number of colonies >100 µm observed in cells expressing pcDNA, pcDNA-Meq, or pcDNA-MeqFos. Average number of colonies counted from three random fields are shown. Error bars indicate SD.

3B, C and D respectively). In addition, DNA binding of Meq and MeqFos to the *meq* promoter and 3X-Ori was further confirmed by chromatin immunoprecipitation (ChIP) assay (data not shown).

Colony formation in soft agar

Colony formation in soft agar is a marker of transformation, and Meq expression in fibroblasts has been shown to promote anchorage independent growth (Levy et al., 2005). Soft agar assay was employed to test the ability of Meq heterodimers to promote colony formation in soft agar. Meq and MeqFos expression in selected DF-1 cells was confirmed by IFA (Fig. 4A top panel). As expected, selected DF-1 cells expressing the pcDNA-Meq construct formed large colonies in soft agar when compared to control cells selected with pcDNA empty vector (Fig. 4A bottom panel). Furthermore, cells expressing pcDNA-Meq had 25× more colonies, >100 µm in size, compared to pcDNA control cells. In contrast, DF-1 cells expressing pcDNA-MeqFos only had 3.5× as many colonies >100 µm in size compared to empty vector control cells (Fig. 4B), indicating that MeqFos heterodimers have a reduced potential to confer anchorage independent growth.

Construction of a Meq-heterodimer mutant virus rMd5-MeqFos

A recombinant Md5 mutant virus in which the leucine zipper region of *meq* was replaced with the corresponding region of *Fos* (rMd5-MeqFos) was successfully constructed. The leucine zipper region of the *meq* gene was replaced with the leucine zipper region of *Fos* by overlapping PCR, and the EcoQ fragment containing the chimeric *meqFos* gene was cloned into the A6Δmeq and SN5Δmeq cosmids. The resultant cosmids A6-MeqFos and SN5-MeqFos together with cosmids P89, B40 and SN16 were cotransfected into DEF by the calcium phosphate method and a recombinant virus was subsequently recovered by homologous recombination. Southern blot analyses were performed to assess the integrity of the rMd5-MeqFos viral genome. Genomic DNA from rMd5 and rMd5-MeqFos infected DEF was digested with *Eco*RI and probed with ^{32}P labeled Md5 comids DNA (Fig. 5A) or digested with *Pst*I and probed with ^{32}P labeled EcoQ fragment DNA (Fig. 5B). No differences were observed between rMd5 and rMd5-MeqFos digestion patterns confirming the gross integrity of the recombinant genomes (Fig. 5). A *Pst*I site is located in the leuzine zipper region of both rMd5 and rMd5-MeqFos, therefore two bands (3950, 2529 base pairs) are observed in DNA digested with *Pst*I and probed with EcoQ fragment DNA. Furthermore, the expression of Meq by rMd5 and rMd5-MeqFos viruses in infected DEF was confirmed by IFA (Fig. 6A).

In vitro and in vivo replication of rMd5-MeqFos

The *in vitro* growth properties of rMd5-MeqFos were tested, to assess if the leucine zipper mutations had any effect on virus replication *in vitro*. Our results indicate that rMd5 and rMd5-MeqFos virus replicated similarly at the time points tested (days 0, 2, 4, 6) (data not shown).

To test the role of Meq heterodimers during *in vivo* replication, 4-week-old SPF chickens were inoculated with 3000 pfu of rMd5 or rMd5-MeqFos. Six days after inoculation two randomly selected birds were euthanized and lymphoid organs (thymus, spleen and bursa of Fabricius) collected and evaluated for viral lytic antigen (pp38) expression by IHC. Expression of pp38 was present in bursa of Fabricius (Fig. 7) thymus and spleen (data not shown) of both rMd5 and rMd5-MeqFos infected chickens, indicating that the leucine zipper mutations in the rMd5-MeqFos virus did not affect early cytolytic infection.

GaHV-2 switches from an active cytolytic infection to a latent infection approximately 7–8 days PI and virus reactivation can be observed when latently infected lymphocytes are co-cultured with fibroblasts (Calnek, 1986). To examine if Meq heterodimers are involved in the establishment of latency and reactivation, peripheral

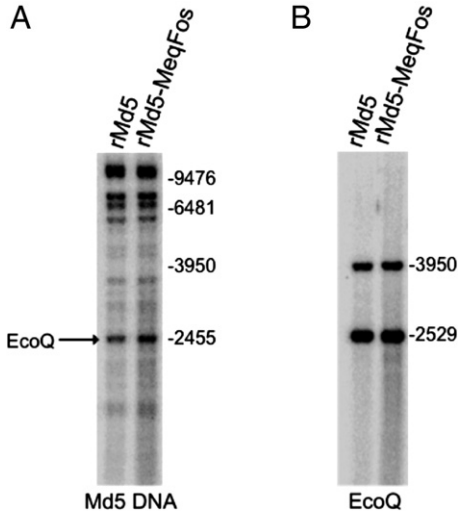

Fig. 5. Southern blot analysis of recombinant viruses genomic DNA. (A) rMd5 and rMd5-MeqFos DNA was digested with *Eco*RI and probed with total viral MDV cosmid DNA. (B), rMd5 and rMd5-MeqFos DNA was digested with *Pst* and probed with EcoQ fragment. The restriction profile of rMd5-MeqFos is similar to that of rMd5, indicating no gross genome rearrangements incurred.

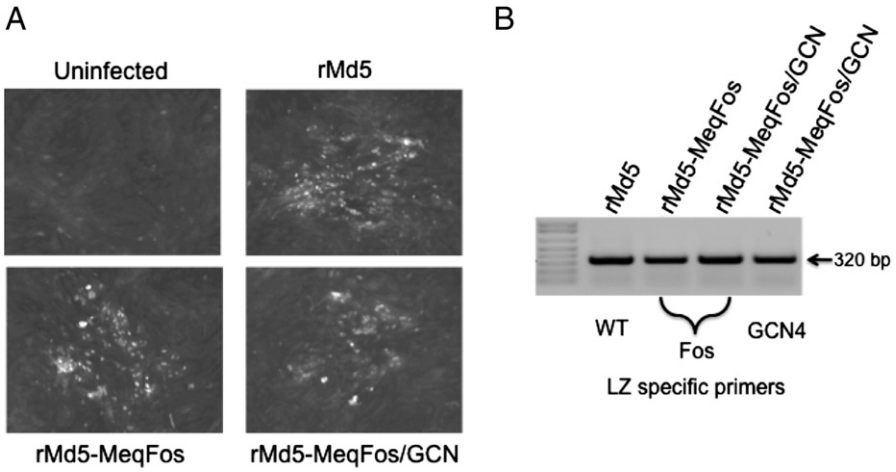

Fig. 6. Expression of Meq in DEF cells infected with recombinant viruses. (A) Uninfected DEF, rMd5-Meq, rMd5-MeqFos and rMd5-MeqFos/GCN infected DEF were stained with rabbit anti-Meq specific antibodies (100× magnification). (B) RNA isolated from rMd5, rMd5-MeqFos, and rMd5-MeqFos/GCN infected DEF was examined for *meq* transcription by RT-PCR with Meq and leucine zipper specific primers. The expected 320 bp amplicon was observed in all three viruses examined.

blood lymphocytes (PBMC) were obtained from three chickens from each group on days 14 and 21 PI (experiment 1) and at day 37 PI (experiment 3) and co-cultured with DEF. As shown in Table 1, none or few viral plaques were obtained from PBMC of chickens infected with rMd5-MeqFos at days 14 days and 21 PI; however, viral antigen expression in PBMC was confirmed by IFA (data not shown). Interestingly, reactivation levels comparable to rMd5 were obtained at day 37 PI from one out of three chickens in rMd5-MeqFos infected chickens (Table 1). To assess if any mutations in the MeqFos gene incurred, which may have contributed to higher reactivation numbers observed, the MeqFos gene was sequenced from recovered viruses but no mutations were detected (data not shown).

Transmission of GaHV-2 occurs after viral replication in feather follicle epithelium (FFE) and release of infectious dander (Calnek, 2001). Therefore, to assess if the rMd5-MeqFos heterodimer mutant was able to replicate in the FFE, expression of the lytic viral antigen pp38 was evaluated in three chickens from each inoculated group by IHC. Tissues from both rMd5 and rMd5-MeqFos infected groups tested positive for pp38 antigen indicating the ability of rMd5-MeqFos, like rMd5, to replicate in the FFE (Fig. 7). To further evaluate virus transmission, day old SPF chickens were inoculated with 3,000 pfu of rMd5 or rMd5-MeqFos and housed with three uninfected contact chicks. Buffy coats were collected from contact chickens 8 weeks PI, DNA extracted and PCR for Meq performed. Transmission to contact chickens was confirmed by PCR in two chickens infected with rMd5 and all three contact chickens infected with rMd5-MeqFos were PCR positive for *meq* (data not shown). Altogether these results confirm that rMd5-MeqFos retains the ability to transmit horizontally by shedding through the FFE.

Oncogenecity of rMd5-MeqFos

To determine if Meq-Jun heterodimers are sufficient for *in vivo* transformation of T-cells, groups of ten SPF chickens were inoculated at day of age with 2,000 pfu of rMd5 or rMd5-MeqFos and observed for mortality for a period of 8 weeks. All chickens that died during the experiment or were euthanized at the termination of the experiment were evaluated for MD-specific lesions. MD-associated mortality was observed in rMd5-infected chickens starting at week 4 PI and none of the chickens survived to the end of the experiment. Only preneoplastic lesions were observed in the rMd5-MeqFos group (Fig. 8) and paralysis was not observed in any of the rMd5-MeqFos infected chickens.

Oncogenecity of rMd5-MeqFos/GCN

To complement our current study, investigating the role of Meq-Jun heterodimers in transformation, we recovered a virus, rMd5-MeqFos/GCN, which contains one copy of the mutant *MeqGCN* gene and one copy of the *MeqFos* gene. Meq expression by rMd5-MeqFos/

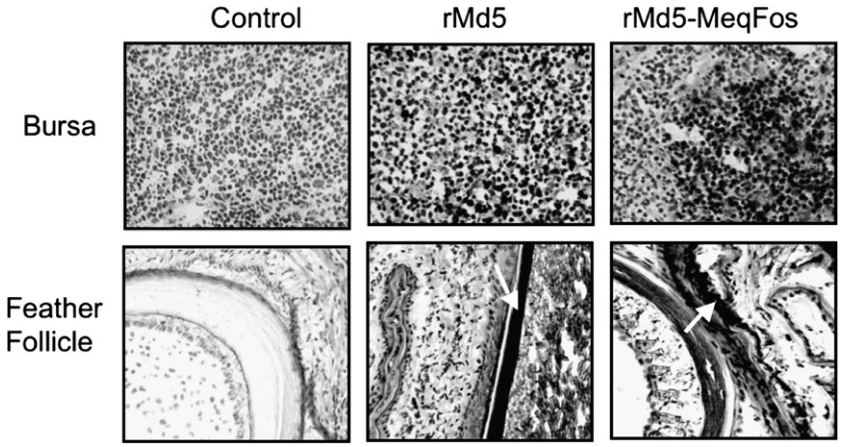

Fig. 7. Infection of lymphoid tissue and feather follicles of chickens infected with rMd5 and rMd5-MeqFos. IHC of bursa of Fabricius and feather follicles, 6 and 21 dpi, respectively, was carried out using anti-pp38 monoclonal antibody and counterstaining was performed with hematoxylin. Positive cells are indicated by black staining.

Table 1
Virus reactivation from peripheral blood lymphocytes.[a]

	Days post inoculation		
Group	14	21	37[b]
rMd5	121	28	116.5
	181	120	118.5
	75	123	129
rMd5-MeqFos	0	0	2.5
	1	0	8
	5	0	133.5
rMd5-MeqFos/GCN	ND	ND	1.5
			4
			158

[a] Numbers represent the average number of pfu observed when 10^6 peripheral blood lymphocytes were co-cultured with DEF form each chicken in duplicate.
[b] Samples were collected from chickens from experiment 2.

GCN in infected DEF was confirmed by IFA (Fig. 6A). In addition, due to lack of Fos and GCN4 leucine zipper specific antibodies, transcription of both MeqFos and MeqGCN in DEF infected with rMd5MeqFos/GCN was confirmed by RT-PCR (Fig. 6B).

To study if both Meq homodimers and Meq-Jun heterodimers contribute to GaHV-2 transformation, ten SPF chickens were inoculated at day of age with 2000 pfu of rMd5-MeqFos/GCN. Preneoplastic lesions were observed in 60% of chickens and paralysis and neoplastic lesions in 20% of chickens infected with rMd5-MeqFos/GCN (Fig. 8, Table 2). In addition, one out of three contact chickens from the rMd5-MeqFos/GCN infected group contained preneoplastic nerve lesions.

Construction and biological properties of revertant virus rMd5-MeqFosR

To verify that the phenotypic changes observed with rMd5-MeqFos were due to changes introduced in the leucine zipper region, a revertant virus was constructed. DNA obtained from rMd5-MeqFos infected CEF was co-transfected with purified parental GaHV-2 EcoQ DNA, which contains the *meq* gene. A revertant virus, rMd5-MeqFosR, was selected by plaque purification, and screened for the presence of both parental *meq* and mutant *meqFos* genes by PCR. PCR revealed that of the 102 plaques tested, one contained one copy of the parental *meq* gene restored in the viral genome. Three chickens were infected with 3,000 pfu of rMd5-MeqFosR and by week 5 PI all three were positive for MD gross tumors, confirming that the attenuated

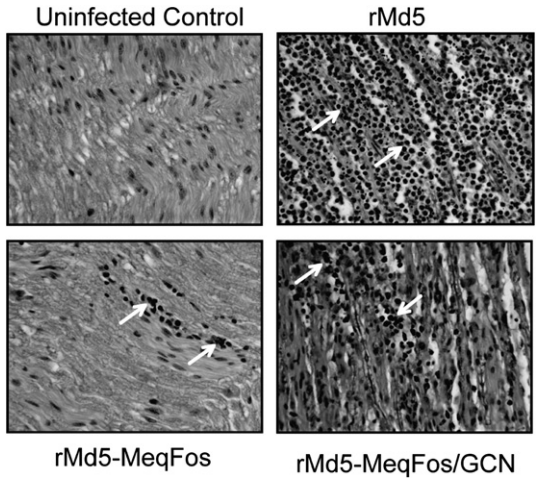

Fig. 8. MDV induced preneoplastic and neoplastic nerve lesions in chickens infected with rMd5, rMd5-MeqFos and rMd5-MeqFos/GCN. Neoplastic nerve lesions were observed in rMd5 (diffuse infiltration, arrows) and rMd5-MeqFos/GCN (arrows) infected chickens while preneoplastic lesions (arrows) were observed in chickens infected with rMd5-MeqFos.

Table 2
Pathogenicity of rMd5, rMd5-MeqFos, rMd5-MeqFosR and rMd5-MeqFos/GCN in SPF MDV maternal antibody-negative chickens.

Virus	MDV mortality (%)	Histological lesions (%)	
		Preneoplastic	Neoplastic
rMd5	10/10 (100)	ND	ND
rMd5-MeqFos	0/10 (0)	4/8 (50)	0/0 (0)
rMd5-MeqFosR[a]	3/3 (100)	ND	ND
rMd5-MeqFos/GCN	2/10 (20)	6/10 (60)	2/10 (20)
Uninfected	0/8 (0)	0/3 (0)	0/3 (0)

[a] Performed as an independent experiment.

phenotype of rMd5-MeqFos was due to the mutations introduced in the leucine zipper of Meq.

Discussion

In this study, the role of Meq-Jun heterodimers in GaHV-2 transformation was investigated to complement our recent investigations on the role of Meq/Meq homodimers in pathogenesis (Suchodolski et al., 2009). We previously showed that rMd5-MeqGCN, a recombinant Meq homodimer, is non-oncogenic, supporting the concept that Meq/c-Jun heterodimers play a role in transformation. The results of the current study further dissect the functions of Meq homodimers and heterodimers in GaHV-2 pathogenesis and we demonstrate that together both Meq homodimers and heterodimers contribute to transformation of chicken lymphocytes.

In order to assess the functions of Meq-Jun heterodimers in GaHV-2 pathogenesis, a recombinant virus in which the leucine zipper of the Meq protein was replaced with the leucine zipper of c-Fos was successfully generated. We took advantage of the fact that the leucine zipper of c-Fos exclusively heterodimerizes with the Jun family of transcription factors (Kouzarides and Ziff, 1989; Ransone et al., 1990; Vogt, 2001) and generated a MeqFos chimeric gene. We also generated an additional virus containing one copy of the previously described MeqGCN (homodimer) gene and one copy of MeqFos (heterodimer) to evaluate the combined transforming capacity of both Meq mutant genes.

The role of Meq-Jun heterodimers in rMd5 pathogenesis was investigated by evaluating both viral replication, latency/reactivation and transformation properties of rMd5-MeqFos *in vivo*. *In vitro,* rMd5-MeqFos replicated similar to parental virus (data not shown) and *in vivo,* early cytolytic infection was similar to parental virus in lymphoid organs as assessed by expression of lytic protein pp38 (Fig. 7). As the FFE is the site of fully productive infection and source of horizontal transmission (Calnek, Adldinger, and Kahn, 1970), we evaluated replication of rMd5-MeqFos in the FFE by IHC and transmission to contact chickens. Our results show that Meq heterodimerization did not interfere with either replication in the FFE or transmission to contact chickens. These results are not unexpected as Meq is not required for replication in lymphoid organs or FFE (Lupiani et al., 2004).

We previously reported the rMd5-MeqGCN appeared to be defective in reactivation and speculated, therefore, that Meq heterodimers may play a role in reactivation from latency (Suchodolski et al., 2009). The switch from lytic to latent infection in GaHV-2 occurs around 7 days PI, and virus from latently infected cells can be reactivated when co-cultured with fibroblasts *in vitro* (Calnek, 1986). In reactivation assays, rMd5-MeqFos, appeared to have impaired reactivation as none or few viral plaques were obtained at days 14 and 21 PI (Table 1) even though viral antigen expression in PBMC was confirmed by IFA. Intriguingly, our IFA results show viral pp38 expression was only detected in dividing PBMC. This observation is similar to latent infection with EBV in Burkitt's lymphoma where viral antigen expression was only detected in dividing memory B-cells thus supporting a true latent infection (Hochberg et al., 2004). These

results do not support that Meq/c-Jun heterodimers are involved in reactivation. However, since our assessment was not quantitative, we cannot dismiss the possibility of decreased viral loads as was observed in leucine zipper mutants (Brown et al., 2009). Interestingly, at day 37 PI one chicken from rMd5-MeqFos and rMd5-MeqFos/GCN had viremia comparable to parental rMd5 but a correlation between high reactivation levels and neoplastic lesions was not evident (Table 1). Therefore, we can only speculate that host factors may have contributed to higher reactivation levels in these two chickens.

In order to assess the role of Meq-Jun partnership in GaHV-2 transformation *in vivo*, SPF chickens were infected with rMd5-MeqFos and evaluated for MD specific lesions for an eight-week period. In agreement with a previous report (Brown et al., 2009), neoplastic lesions did not develop in chickens infected with rMd5-MeqFos suggesting that heterodimerization of Meq alone is not sufficient for transformation of lymphocytes in GaHV-2 infected chicken. In addition, the revertant virus, rMd5-MeqFosR, generated in our study showed no difference in pathogenesis, in terms of tumor incidence or survival time, with parental rMd5 suggesting that the observed phenotype was due to mutations introduced to confer heterodimerization and not due to additional mutations at secondary sites. In contrast, the revertant viruses generated by Brown et al. did not fully restore the pathogenic phenotype (e.g. only 40–60% mortality compared to 100% for parental virus up to 70 dpi) suggesting that secondary mutations might have affected the phenotype observed (Brown et al., 2009).

To study if both Meq homo and heterodimers are involved in complementing pathways in GaHV-2 pathogenesis, an additional virus, rMd5-MeqFos/GCN, which contains one copy of MeqFos and one copy of MeqGCN was generated. Neoplastic lesions and paralysis were observed in 20% of chickens infected with rMd5-MeqFos/GCN. These data strongly support that both Meq homo- and heterodimers are involved in complementing pathways leading to T-cell transformation. However, rMd5-MeqFos/GCN clearly displayed a phenotype distinct from rMd5, as visceral tumors were not observed and MD mortality was significantly reduced. It is possible that interactions of Meq with bZIP proteins other than c-Jun could contribute to GaHV-2 pathogenesis. For example, Meq has been shown to dimerize with ATF/CREB family members (Levy et al., 2003), which have been implicated in oncogenesis in other systems (Siu and Jin, 2007; Vlahopoulos et al., 2008). In addition, the interaction of Meq with other unknown cellular factors may have been abrogated by replacement of the parental leucine zipper with Fos and GCN leucine zippers contributing to decreased transformation. Another possible explanation for the loss of oncogenicity could be that the mutations introduced into the Meq lecine zipper could have affected the generation of *meq* splice variants and *meq* antisense open reading frames observed in wild type viruses (Anobile et al., 2006; Liu, Soderblom, and Goshe, 2006; Peng et al., 1992; Peng and Shirazi, 1996a, 1996b; Peng et al., 1995). However, the role of these *meq* spliced variants and antisense open reading frames as well as Meq interactions with host factors remains to be determined.

Our *in vitro* characterization of MeqFos also revealed that under physiologic conditions MeqFos is recruited to the GaHV-2 Ori and represses the bidirectional pp38/pp14 promoter (Fig. 3B, C, D). Therefore, loss of oncogenecity by MeqFos is not a result of lack of ability to repress pp38/pp14 promoter. These results are in disagreement with those previously reported by EMSA (Brown et al., 2009; Levy et al., 2003). It is therefore possible that the presence of unknown cellular factors in the luciferase and Chip assay could contribute to recruitment of MeqFos to these promoters under physiologic conditions, compared to EMSA analysis that utilized bacterially expressed truncated Meq proteins.

In summary, this work provides the first *in vivo* evidence that both Meq homodimers and Meq-Jun heterodimers are involved in complementing pathways required for GaHV-2 transformation. Since, rMd5-MeqFos/GCN was attenuated compared to parental rMd5, Meq heterodimerization with other AP-1 members outside the Jun family such as ATF/CREB may also contribute to GaHV-2 transformation. Further work investigating the role of Meq and ATF/CREB members will help in developing a better understanding of the transforming pathways in GaHV-2 pathogenesis.

Materials and methods

Cells and viruses

Primary duck embryonic fibroblasts (DEF) were used for virus propagation, virus reactivation assay, growth curves and DNA transfections. Cosmids derived from a very virulent GaHV-2 strain, Md5 were used to generate recombinant viruses (Reddy et al., 2002). Revertant virus (rMd5-MeqFosR) was recovered in chicken embryonic fibroblasts (CEF). The DF-1 cell line was used for luciferase and chromatin immunoprecipitation (ChIP) assays (Kim et al., 2001). Co-immunoprecipitation (co-IP) experiments were performed using 293 T cells. Both DEF and CEF were maintained in Leibowitz–McCoy (LM 1:1) media supplemented with 5% bovine calf serum (BCS) and penicillin-streptomycin at 37 °C. DF-1 cells were maintained in LM media supplemented with 4% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C. 293 T cells were maintained in DMEM media supplemented with 10% FBS and penicillin-streptomycin at 37 °C.

Generation of mutant MeqFos chimeric gene

Mutations in the *meq* gene were generated as described previously (Suchodolski et al., 2009) with the exception that the primers used for overlapping PCR were specific for the *c-Fos* leucine zipper. Briefly, *meq-Kpn*I, which is located within the EcoQ fragment of the Md5 genome and contains the coding sequence for *meq* nucleotides 1–385, was mutated by a series of subcloning steps and overlapping PCR. Overlapping PCR was performed in a combination of three PCR reactions to replace the leucine zipper region of *meq* with the leucine zipper region of *c-Fos*. Two primary PCR reactions were performed to generate the 5′ and 3′ ends of *meqFos-Kpn*I fragment. The 5′ reaction was performed using primers M13R (5′-CAG GAA ACA GCT ATG AC-3′) and SR1194-*Fos* Leucine Zipper Reverse (5′-CTC CGC CTG CAG AGC GGA CTT CTC CTC CTC CAG CTG GTC CGT CTC CGC CTG CAG TTT GTC TAC ATA GTC CGT CTG CTT CCT-3′). The 3′ end PCR reaction was performed using primers SR1190 (5′-GAC CGA GAT AGG GTT GAG TG-3′) and SR1195-*Fos* Leucine Zipper Forward (5′-GAG AAG TCC GCT CTG CAG GCG GAG ATA GCC AAC CTG CTG AAG GAG AAG GAG AAG CTG CGT GTA CAG TTG GCT TGT CAT GAG CCA-3′). Both amplicons were gel purified, mixed together and used as templates in a third PCR reaction with primers M13R and SR1190, generating a full *meq-Kpn*I fragment containing the *Fos* leucine zipper in place of the *meq* leucine zipper (*meq-Fos-Kpn*I). The *meqFos-Kpn*I fragment was cloned into the EcoQ fragment, generating EcoQ-MeqFos.

Co-Immunoprecipitation

Co-immunoprecipitation analyses were performed to evaluate MeqFos heterodimerization. For *in vitro* heterodimerization assays, parental *meq*, *MeqFos*, and chicken c-*Jun* were cloned into the pcDNA3.1 (Invitrogen, Carlsbad, CA) eukaryotic expression vector. *Meq*, *MeqFos, and c-Jun* genes were cloned in frame with HA and Flag tags to aid in protein detection. The resulting plasmids were denoted as pHA-MeqFos, pFlag-MeqFos, pHA-Meq, pFlag-Meq, and pJun-HA.

293 T cells were cotransfected with 2 μg of either pFlag-Meq or pFlag-MeqFos and 2 μg of pHA-Meq, pHA-MeqFos, or pJun-HA using Fugene 6 (Roche, Mannheim, Germany). Cells were harvested 48 h after transfection and lysed in TNE buffer (10 mM Tris (pH 7.5), 150 mM NaCl, and 0.2 mM EGTA) containing 1 mM DTT. Cell lysates

(500 µl) were immunoprecipitated with 20 µl of Flag mouse antibody-conjugated agarose beads (Sigma, St. Louis, MO). Immune complexes were captured with 20 µl of a protein A and protein G sepharose bead mixture and rocked for an additional 2 h at 4 °C. Beads were washed four times with TNE buffer and boiled for 5 min in 20 µl of 2× sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris–HCl [pH 6.8], 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.6% bromphenol blue) subjected to SDS-PAGE electrophoresis and transferred to PVDF membranes. After blocking for 1 h at room temperature (RT) with 5 % skim milk in TBST (20 mM Tris–HCl [pH 7.5], 137 mM NaCl, 0.05% Tween 20), the membranes were incubated with a 1:500 dilution of anti-HA tag antibody (Convance, Berkley, CA) for 2 h at RT. The membranes were washed with TBST three times for 10 min each at RT, and incubated with horseradish peroxidase-conjugated antibodies for 1 h at RT. Membranes were washed three times with TBST and visualized with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

Luciferase assays

The transactivation/repression activity of the MeqFos protein on GaHV-2 promoters was evaluated by luciferase assay. The previously described *meq*, pp38, pp14 and GaHV-2 Ori promoter constructs (Suchodolski et al., 2009) were used for luciferase assays. *Meq*, *MeqFos*, and *c-Jun* genes were cloned into the pcDNA 3.1 Zeo vector (Invitrogen) to generate expression vectors, pcDNA-Meq, pcDNA-MeqFos, and pcDNA-Jun. DF-1 cells (1×10^5) were seeded in 12-well plates 16 h prior to transfection and incubated at 37 °C under standard conditions. Transfections were performed using siPORT XP-1 (Ambion, Austin, TX), 500 ng of pcDNA (empty vector control), pcDNA-Meq, pcDNA-MeqFos, or 250 ng of pcDNA-Jun with either 250 ng of pcDNA-Meq or pcDNA-MeqFos, and either 250 ng of pGL3-*meq*, pGL2-Ori, or pGL3-*pp14* or pGL3-*pp38* promoters. Cell lysates were harvested 48 h post transfection with active lysis buffer (Promega, Madison, WI) and luciferase assay performed using a Biotek Clarity luminometer (Biotek, Winooski, VT). The protein concentration in each transfected sample was measured by Bradford assay (Bio-Rad, Hercules, CA) and luciferase activity was normalized to protein concentration. Assays were performed in triplicate and three independent experiments were performed for each reporter vector tested. Transactivation or transrepression activity was expressed as the fold differences relative to empty pcDNA vector control. Results of all three experiments were analyzed by one-way ANOVA followed by Tukey HSD test using the SPSS®, Version 14.0 software (SPSS Inc., Chicago, IL, USA). For all analysis, $p \leq 0.05$ was considered statistically significant.

Colony formation in soft agar

DF-1 cells were transfected with pcDNA, pcDNA-Meq or pcDNA-MeqFos using siPORT XP-1 (Ambion). Approximately 48 h after transfection, the transfected DF-1 cells were selected with 300 µg/ml zeocin (Invitrogen) for 4 weeks. Expression of Meq was confirmed by IFA using rabbit anti-Meq antibodies as described below. Pools of resistant cells (5×10^3) were seeded in 0.33% agarose containing LM media with 150 µg/ml zeocin and 10% FBS overlaid on a 0.5% agarose in a 35 mm plate. After 3 weeks of culture, colonies were examined under a light microscope and photographed using 12× magnification. Three different fields were randomly selected and colonies greater than 100 µm were counted. Two independent experiments were performed and each experiment was performed in triplicate.

Cosmids

Previously described cosmids, SN5, P89, SN16, A6 and B40, encompassing the entire genome of the very virulent strain Md5 (Reddy et al., 2002) were used to generate a recombinant Md5 (rMd5) virus and a recombinant Md5 with a chimeric Meq gene containing the leucine zipper from c-Fos (rMd5-MeqFos) (Fig. 1). The EcoQ-MeqFos generated as indicated above was subsequently cloned into cosmids A6ΔEcoQ and SN5ΔEcoQ using recA assisted restriction endonuclease digestion (RARE) yielding cosmids SN5-MeqFos and A6-MeqFos.

Transfections

Viral inserts were released from parental and mutant cosmid DNA by digestion with *Not*I and purified by phenol chloroform extraction and ethanol precipitation before transfection. The calcium phosphate procedure was used to transfect 1.2 x10^6 DEF in 60 mm dishes with 500 ng of cosmids P89, SN16, and B40 in combination with SN5 and A6, or SN5-MeqFos and A6-MeqFos, or SN5-MeqFos and A6-MeqGCN, to generate rMd5, rMd5-MeqFos, and rMd5-MeqFos/GCN, respectively. Five days after transfection, cells were trypsinized and seeded onto fresh DEF monolayers and monitored for cytopathic effect. Viral stocks of recovered viruses were subsequently made and titrated in DEF for further analysis.

Southern blot

DNA from rMd5 and rMd5-MeqFos infected DEF was isolated by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Three micrograms of each DNA sample was digested with *Eco*RI or *Pst*I, separated on a 1% agarose gel, and transferred to nylon membranes. ^{32}P-dCTP-labeled probes representing the complete GaHV-2 genome (cosmids SN5, P89, SN16, A6 and B40) or EcoQ fragment (2,456 bp) were generated by random priming, using High Prime DNA Labeling Kit (Roche), and were hybridized to digested viral DNA, using standard protocols (Sambrook, 2001).

RT-PCR

RT-PCR was performed to confirm expression of *meqFos* and *meqGCN* genes in DEF infected with rMd5-MeqFos/GCN. Total RNA was extracted using RNAqueous®-4PCR (Ambion). The reverse transcriptase reaction was performed using equal amounts of RNA followed by PCR. Four µl of cDNA were used for subsequent PCR reactions using primers SR1118-*meq* start primer (5′-GAT CCC GGG GAG ATG TCT CAG GAG CCA GAG C-3′) and leucine zipper specific primers SR3075-*Fos* leucine zipper (5′-TTC AGC AGG TTG GCT ATC-3′), or SR3074-*GCN4* leucine zipper (5′-CTA ATT TCT TTA ATC TGG CAA C-3′).

Generation of revertant virus

CEF were inoculated with rMd5-MeqFos to obtain viral DNA used for transfections. Gel purified parental Md5 EcoQ fragment together with proteinase K digested and phenol-chloroform extracted rMd5-MeqFos genomic DNA, were used to co-transfect CEF by the calcium phosphate procedure. After viral plaques were evident, individual viral plaques were isolated as described (Suchodolski et al., 2009).The presence of parental *meq* or *Fos* leucine zipper sequences from individual plaques were detected by PCR using the following primers: SR1118-*meq* start primer and leucine zipper specific reverse primers SR3073-*meq* leucine zipper (5′-GTC CTT AGA TCT CGA ATT TCC-3′) or SR3075-*Fos* leucine zipper, for parental and *Fos* leucine zippers, respectively.

Indirect immunofluorescence assay (IFA) and immunohistochemistry (IHC)

Confluent DEF monolayers were infected with rMd5 or rMd5-MeqFos, and when viral plaques were apparent, cells were fixed with ice-cold acetone/alcohol (6:4) and Meq expression evaluated by IFA

using rabbit polyclonal anti-Meq antibodies (1:300). For IHC, lymphoid organs (thymus, spleen and bursa of Fabricius), and feather follicles from infected and uninfected chickens were embedded in optimal cutting temperature compound (Tissue-Tek, OCT, Sakura Finetek, Torrance, CA), immediately frozen in liquid nitrogen and stored at −80 °C until use. Six- to 8-μm-thick cryostat sections of tissue were prepared and fixed with cold acetone at −20 °C for 5 min, and air-dried. Immunostaining was carried out using H19 pp38 monoclonal antibody (1:3000) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Pathogenesis experiments

Experiment 1

To study the effect of Meq heterodimers on viral replication, 4-week-old SPF chickens (Hy-Vac, Adel, Iowa) were randomly sorted into experimental groups of nine chickens each. One group remained as a non-inoculated control, whereas the other groups were inoculated subcutaneously with 3,000 PFU of rMd5 or rMd5-MeqFos. At 14 and 21 days post infection (dpi), blood samples, from three randomly selected chickens, were collected in heparin for reactivation assays (see below), and were subsequently euthanized for tissue sample collection. At 6 and 21 dpi lymphoid organs and feather follicles were collected from two and three chickens, respectively, for IHC.

Experiment 2

To study the role of the Meq heterodimers on oncogenesis, SPF day old chicks (ten per group) were inoculated subcutaneously with 2,000 PFU of rMd5, rMd5-MeqFos, or rMd5-Meq/Fos/GCN and reared in modified Horsfall–Brauer isolation units for 8 weeks. Weekly mortality was recorded and all chickens were necropsied at time of death or at termination of the experiments (8 weeks) and evaluated for MD specific lesions in the viscera and the nerves. Blood was also collected from three randomly selected chickens from each group for reactivation assays at day 37 dpi.

Experiment 3

To study the role of Meq heterodimers on horizontal transmission, six SPF (Charles River, Wilmington, MA) day old chicks were inoculated subcutaneously with 3,000 PFU of rMd5 or rMd5-MeqFos while three additional uninoculated chicks were reared with each group and served as contacts to assess horizontal transmission. At 8 weeks post inoculation buffy coats were obtained from heparinized blood by centrifugation at 500×*g*. DNA was extracted using Purelink Genomic DNA extraction kit (Invitrogen) and PCR was performed using GaHV-2 specific Meq primers SR1118 (5′-GAT CCC GGG GAG ATG TCT CAG GAG CCA GAG C-3′) and SR1135 (5′-GAT CCC GGG TCA GGG TCT CCC GTC ACC TGG AAA CC-3′) to detect the presence of viral genome.

Reactivation assay

Buffy coats were collected as described above at 14, 21, and 37 dpi. DEF monolayers seeded in 35 mm plates were inoculated with 10^6 lymphocytes in duplicate and viral plaques counted 7 days post inoculation.

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